ANTIBODY PROFILES IN CASES OF VIVAX MALARIA

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This is to certify that the work presented in this dissertation was carried out by **Ms. Huma Umar** in my laboratory. It is suitable for the award of the degree of Master of Philosophy (M.Phil) in Microbiology.

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ABSTRACT

Serological assessment was carried out to construct the antibody profiles in vivax malaria in a population sample comprising of 63 individuals belonging to different age groups from the Aligarh District - an area endemic for malaria. Indirect fluorescent antibody (IFA) tests were used to detect and estimate the IgG, IgM and IgA antibodies in the sera samples. A titre of 1:20 was taken as an index of an active malaria infection.

On an overall basis, antimalarial IgM levels were found to be higher in most cases of slide-proven vivax malaria, as also in significant amounts in the healthy controls of all age groups. Initial results were fairly suggestive of an active transmission of malaria in the study area.

Antimalarial IgG titres were found to show an increasing trend after 2 years of age. In adults, IgG levels were similar in slide-proven cases of vivax malaria and in healthy controls, indicating not only the prevalence of the disease in the area under investigation but also a significantly higher level of humoral immunity among the resident adults. Presence of antimalarial IgA was also recorded in a few cases, the significance of which was not quite clear on the basis of this investigation. IgM levels

were found to be less affected by chemotherapy as compared to IgG levels. The significance of this finding, in relation to the serological status of the individuals exposed naturally to malaria, has been discussed in the appropriate section of this dissertation.

INTRODUCTION

Malaria is a disease caused by sporozoa of the genus Plasmodium. It is characterized by fever, which is often periodic, chills and sweating, varying degrees of anaemia, splenic enlargement, and various syndromes resulting from the physiological and pathological involvement of certain organs including the liver and the kidneys (1).

Malaria remains the most important of all the parasitic infections in terms of global impact and it is a threat to almost half the world's population. Malaria is widely endemic in the less developed countries in the tropics, where children who constitute up to half of the population are beset with all manner of insults to their health and well-being that start prenatally and continues throughout the childhood (2). In India it is universally recognized as one of the most important health problems and constitutes an adverse factor in the social and economic advancement of the country (3,4).

1.1 HISTORICAL REVIEW

Human malaria is probably as old as mankind. Garnham in 1966 (5) considered the collateral evolution of malarial parasites of the primates with that of their hosts, starting with their vertebrate hosts. The first prosimian primates evolved some 50 million years ago. These lemur-

like animals were probably associated with the appearance of Hepatocystis and eventually with the quartan groups of the malarial parasites. Over a period of a million years series of evolutionary changes occurred in the original quartan group of malarial parasites and between 10 and 2 million years ago the present sub-genus Laverania emerged.

Fossil mosquitoes were found in geological strata 30 million years old and there is no doubt that they have spread infection throughout the warmer regions of the globe, long before the dawn of history (6).

Prehistoric man in the Old World was subject to malaria. It is probable that the disease originated in Africa which is believed to be the cradle of the human race. Malaria followed with the wake of human migrations to the Mediterranean shores, to Mesopotamia, the Indian peninsula and South East Asia. How malaria established itself in the New World is still a subject of speculation (7).

The earliest indications of a human disease suggestive of malaria came fom ancient Egypt. The consensus is that in prehistoric times, malaria was very common in the southern valley of the Nile, accessible from the vast hinterland of Tropical Africa. Enlarged spleens probably due to malaria have been found in mummies about 3000 years old and splenomegaly with fever has been mentioned in Ebers

Papyrus of 1570 BC (7).

Hippocrates in 400 BC (8) gave the first accurate clinical description of the disease. He had for the first time mentioned the classical triad of chills, fever and sweating and some complications of the disease. He further analysed the characterstic periodicity of various forms of malaria and associated splenomegaly with the endemicity of malaria and its topographical aspects (9).

References to seasonal and intermittent fever exists in the ancient Assyrian, Chinese and Indian religious and medical texts. Non-medical writers alluded to fevers that affected those who lived in marshy areas. These specific fevers known in England as "agues" received in the 18th century the Italian name "mal'aria" meaning bad air, since it was widely believed that their cause was related to foul air common near the marshy areas. The French term "paludisme" indicating a close connection with the swamps came much later (1).

The most important events in the history of malaria took place towards the end of the 19th century when in 1880 Laveran, a French army surgeon first saw and described malaria parasites in human erythrocytes. Soon after that Romanowsky in Russia developed a new method of staining, which coupled with the improvement of microscopes

accelerated and made studies on plasmodia easier (1).

Ronald Ross working in India in 1897 identified mosquitoes as the vectors of malaria parasites. The whole complex picture of the cycle of development of malaria parasites in man and in the Anopheles mosquito became clearer as a result of further studies by the Italians, namely Amigo Bignami, Giuseppe Bastianelli and Battista Grassi in 1898-99. Confirmation of the fact that malaria is transmitted by female Anopheles mosquito was based on the combined field experiments carried out by Patrick Manson and and his colleagues near Rome and in London in 1900 (10).

During the 20th century much research was devoted to malaria control. Larvicides in the form of oil of Paris green were introduced for preventing the breeding of mosquitoes in stagnant waters. At the beginning of the Second World War, Paul Muller of Switzerland discovered the high insecticidal action of a synthetic compound, dichloro-diphenyl-trichlor-ethane, which was given the abbreviated name, DDT (1).

The first therapeutic advance in malaria occurred at the beginning of the seventeenth century when Juan Lopez a Jesuit missionary recorded the use of the 'Peruvian bark' for treatment of fevers by the Peruvian Indians. In 1742, Carlos Linneaus in Sweden described the tree and gave it the

name Cinchona. Further clinical trials on the use of Cinchona were carried out by Cardinal Juan de Lugo in 1643 in Rome and by Morton and Sydenham in England to prove its efficiency. Pelletier and Caventon in 1820 for the first time succeeded in extracting two alkaloids which they named "quinine" and "cinchonine". Out of these quinine was found to be a more effective antimalarial (1,6,10).

Further efforts were made towards developing cheap and effective drugs against malaria. This was brilliantly accomplished in 1924 by Schulemann's discovery of pamaquine. Subsequently, in 1930 a much more valuable drug- mepacrin was prepared by Kikuth, Mietzch and Mauss. Other valuable synthetic drugs were developed by the Germans, French, Americans and the British in 1934 (Chloroquine), 1945 (Proguanil), 1946 (Amodiaquine), 1950 (Primaquine) and 1951 (Pyrimethamine) (1).

These synthetic drugs were successful due to their potency against malaria parasites and their cheap purchasing cost until the early 60's when the first report of chloroquine resistance against Plasmodium falciparum was reported from Columbia and Brazil. Similar reports of chloroquine resistance came subsequently from various other parts of the world. These findings led to more reseach towards developing alternative effective antimalarials.

Series of drugs were developed including mefloquine in 1971-75, amino-alcohol compounds as possible antimalarials were developed by the United States Walter Reed Institute for Research. In 1979-82 the antimalarial called qinghaosu (artemesinine) was developed followed by the development of a series of other derivatives of 8-amino-quinolines with high activity for treatment of relapsing malaria in 1976-83 (1,10).

Another landmark in the advance of the knowledge of malaria came in 1976 when Trager and Jensen discovered the continuous in vitro culture technique of P. falciparum in the U.S.A. (1).

With the development of the science of biotechnology the isolation of mRNA from P. falciparum and cloning of its DNA in a bacterium (Escherichia coli) was made possible in 1983 (10). The above advances opened up many possibilities for research towards the knowledge of malaria, its treatment, control and finally eradication.

1.2 THE MALARIA PARASITE

The micro-organisms causing malaria are commonly referred to as malaria parasites; this term is restricted to the family Plasmodiidae within the order Coccidiida, suborder Haemosporidiidea which comprises various parasites

found in the blood of reptiles, birds and mammals (1).

The genus Plasmodium is peculiar in :

1. One type of asexual multiplication by division occurring in parenchymal cells of the liver of the vertebrate host (exo-erythrocytic schizogony), and;

2. The mosquito hosts are of various species of Anopheles.

1.2.1 Species and Strains

The family Plasmodiidae contains the single genus Plasmodium, which is divided into nearly 120 species including atleast 22 species found in primate hosts, 19 in rodents, bats or other mammals and about 70 species in birds and reptiles (1).

The plasmodia of the primate hosts are divided into 3 subgroups, within the sub-genus Plasmodium, there are four groups classified mainly according to the periodicity of their erythrocytic schizogony.

There are four generally recognized species of malaria parasites of man; three species of Plasmodium are peculiar to man: P. vivax, P.ovale and P. falciparum. One species is common to man and African apes - P. malariae.

P. malariae - (Laveran, 1881)

P. vivax - (Grassi and Feletti, 1890)

P. falciparum - (Welch, 1897)

P. ovale - (Stephens, 1922)

Finally, several simian species of Plasmodium are rarely found in man including P. knowlesi and P. simian as zoonoses. P. cynomologi and P. bastianelli are identified as accidental laboratory acquired infections while P. inui and P. schwetzi have been known to cause experimental infections (1).

It is not uncommon to observe that some morphological or other characterstics of one well defined species of malaria parasites may vary somewhat from one geographical area to another. The biological variations may be so distinctive that they provide an acceptable basis for referring these parasite populations as strains (1).

1.2.2 Life Cycle

The life cycle of the parasite is essentially similar in all species of plasmodia.

Cycle in man : (schizogony or asexual cycle)

1. *Pre-erythrocytic phase.*

   The parasite is introduced into the body by a bite of the mosquito, as thin, motile, spindle shaped

sporozoites. These stay in the blood circulation for approximately half an hour and then enter the parenchyma cells of the liver. During the succeeding 5-7 days the parasites develop into large multinucleate schizonts which mature and liberate small, round, mononucleate merozoites (1,11,12).

2. Exo-erythocytic phase.

About a decade ago it was discovered that the sporozoites of the relapsing type of malaria, namely P. vivax and P. ovale differentiate either into hypnozoites or into developing tissue schizonts in varying proportion depending on the strain. The hypnozoites remain dormant in the hepatocytes as uninucleated forms for considerable periods. At a predetermined time the hypnozoites begin to grow and undergo exo-erythrocytic schizogony, forming a wave of merozoites that invade the blood, and produce a clinical relapse, although the parasites have been removed from the peripheral blood (1,11,12).

3. Erythrocytic phase.

In this phase, the merozoites enter the red cells and grow as trophozoites. These are more or less rounded bodies, some of which contain a vacuole which displaces the cytoplasm to the periphery, while the nucleus is

situated at the pole. These young parasites are known as the ring forms of the trophozoites. Trophozoites of P. vivax show lively amoeboid movement. At first the trophozoites produce the ring forms, small, round structures clear in the centre with a chromatin dot at one side. These develop into mature trophozoites, large, irregular structures, usually containing dark granules of haemozoin, a product of haemoglobin digestion, occupying most of the red cell volume. When fully developed the nucleus of the trophozoites begins to segment and it becomes a schizont. A mature schizont consists of 6-32 small, round or oval merozites, the number varying with the species. In P. vivax the mature schizont contains an average of 12-18 merozoites. In P. falciparum malaria the infected red cells usually disappear from the peripheral blood before the trophozoites are mature and schizogony occurs in the capillaries of the internal organs. Finally, when the process of schizogony is completed the red cell bursts and merozoites are released into the bloodstream where they attack new red cells and repeat the asexual cycle. The erythrocytic schizogony is repeated over and over again leading to a progressive parasitemia until slowed down by the immune responses of the host or chemotherapy. The paroxysms of fever occur when the merozoites are released in the blood. The morbidity and

mortality in malaria is due to the release of parasitic material and parasites in the erythrocytic phase during the course of the disease (1,11,12).

4. Gametocyte production.

After parasitemia has been present for a few days some merozoites, instead of developing into schizonts, develop into sexual cells or gametocytes. These may be male gametocytes (microgametocytes) or female gametocytes (macrogametocytes). No further development of these cells occurs in the human body. In P. vivax gametocytes may appear in the blood within three days after the appearance of the asexual parasites in the blood. The presence of gametocytes in the blood is essential in rendering the patient infective to the mosquitoes and for the continuity of the life cycle of parasites in the invertebrate hosts (1,11,12).

Cycle in the mosquito (sporogony or sexual cycle)

When a female Anopheles mosquito ingests the blood of the human host with gametocytes in circulation, the red cells set the parasites free in the mosquito's stomach. The macrogametocyte matures to form a female macrogamete while the microgametocyte develops 4-8 flagellum like structures which break off and swim away as male microgametes. The male

and female gametes unite to form a zygote which rapidly becomes a motile cell known as ookinete. This penetrates the muscle of the stomach wall where it becomes a rounded oocyst inside which thousands of spindle shaped sporozoites develop. The cyst finally ruptures liberating the sporozoites into the body cavity from where they find their way into the salivary gland of the female Anopheles which now becomes infective. When the mosquito feeds on the human blood after piercing the skin the sporozoites are injected into the wound and pass into the bloodstream of the vertebrate host, thus continuing the life cycle in man (1,12).

1.3 EPIDEMIOLOGY AND GEOGRAPHICAL DISTRIBUTION

Malaria is one of the most widespread of all parasitic diseases. It is found in regions lying roughly between latitudes 60°N and 40°S. The distribution of the plasmodial species is not uniform. Vivax malaria is widespread in the tropics and subtropics and in some temperate regions, sparing the Negroes of West Africa. It is present in areas of the Middle East, Iran, Pakistan, Bangladesh, India, Sri Lanka, Burma, Thailand, Malaysia, Indonesia, East and Central Africa and Central and South America. Falciparum malaria is found commonly in warm moist climates. It is the dominant form of malaria in the tropical West, Central and parts of East Africa, in regions of the

Middle East, South, Central and Northern India, in parts of Bangladesh and Pakistan and in Burma, Thailand, Laos, Malaysia and Indonesia. It occurs in the Philippines, certain Pacific Islands, Haiti in the Carribean Islands, and in Central and South America. Quartan malaria caused by P. malariae occurs throughout the tropics, chiefly in Africa, South America, India, Sri Lanka and Malaysia. It is not as common as either vivax or falciparum malaria. Ovale malaria is uncommon. It occurs in East, West, and Central Africa, parts of North East Africa, and in South America (13).

1.3.1 Endemicity

Malaria is described as endemic when there is constant incidence of cases over a period of many successive years. Malaria in a community may either be stable or unstable. Stable malaria occurs in regions in which there is a constant, repeated infection. The population has a high degree of immunity and epidemics do not occur. Unstable malaria occurs in regions in which transmission is intermittent, inadequately protected by drug suppression or vector control. In such areas the population has a varying degree of immunity and epidemics are liable to occur (13).

Endemic malaria : may be present in various degrees and the following classification of it is continuously used:

Hypoendemicity : denotes areas where there is little transmission and the effects of malaria are on all the age groups.

Mesoendemicity : is found typically among small rural communities in the subtropical zones with varying intensity of transmission depending on local circumstances.

Hyperendemicity : is seen in areas with intense but seasonal transmission where the immunity is insufficient to present the effects of malaria on all age groups.

Holoendemicity : denotes a perennial transmission of the disease resulting in a considerable degree of immune responses in all age groups, but particularly in the adults.

1.4 CLINICAL DIAGNOSIS OF MALARIA

In the clinical diagnosis of malaria, and of paramount importance, is the eliciting of a geographical history in the routine interrogation of all patients; other points of interest are the ethnic origin of the patients and a history of previous attacks of malaria (4,6).

1.4.1 General Features of an Acute Attack of Malaria

The clinical manifestations of malaria are extremely diverse and may range in severity from a mild headache to the development of acute pulmonary oedema in moribund patients. However, a majority of attacks develop a well recognised pattern in which bouts of fevers alternate with asymptomatic periods. Incubation period varies from species to species. They tend to be between 9 to 14 days in P. falciparum, 12 to 17 days for P. vivax, 30 to 40 days for P. malariae and 13 to 17 days for P. ovale (7).

The classical malaria paroxysm comprises of three successive stages. The **cold stage** starts with rigors and a feeling of intense cold. This stage lasts between 15 to 60 minutes. The **hot stage** follows when the feeling of intense cold gives way to one of distressing heat. The temperature may rise upto 41°C. This stage lasts for 2 to 6 hours. The hot stage is followed by a terminal **sweating stage** when the patient breaks out in profuse sweat, so that his bedding becomes drenched. The temperature falls rapidly, often below normal. This stage lasts from 2 to 4 hours. (1).

Representatives of all development forms of asexual parasites from the early ring to the mature schizont may be recognised in the peripheral blood at any one time, although one stage usually preponderates once the usual

periodicity has been established. The density of parasitemia in vivax malaria seldom exceeds 2% of the infected erythrocytes. Gametocytes may be found after the infection has been present for a week (1,13).

The trend of the classical features are more defined in vivax malaria. The primary attack is composed of a number of paroxysms occurring at 48 hours interval. A single untreated attack of vivax malaria may last for a few weeks to three months or more with repeated paroxysms before spontaneous clinical cure occurs. The duration depends on the multiplication rate of the parasite strain involved and on the counteraction of the immune responses of the host.

1.4.2 Relapses in Vivax Malaria

A relapse is a renewed occurrence of clinical symptoms and parasitemia after a time considerably greater than the intervals between periodic paroxysms of the primary infection. The period during which the malaria infection is not evidenced clinically by any symptoms of disease are known as latency. Parasite latency has been known in vivax malaria in which stage the parasites are absent from the blood but are present as exoerythrocytic forms in the patient's liver. The dormant hepatic forms are known as **hypnozoites** and are the direct descendents of sporozoites which were the precursors of the primary attack of the disease.

In about 60% of untreated or inadequately treated cases of vivax malaria, clinical symptoms recur after a period of quiescence, the length of which depends largely on the particular strain of the parasite. Renewed clinical activity is seen either during the first 8 to 10 weeks after the primary attack, when it is designated as a short-term relapse, or around 30 to 40 weeks after the primary attack when it is referred to as a long-term relapse.

The clinical features of a relapse are similar to those of the primary attack, except that the initial period of irregular fever is absent. The defined periodicity of paroxysms occurring every 48 hours are established from the start. The relapse is commonly less severe and of a shorter duration than the primary attack. It has been suggested that various adverse factors including cold, fatigue, trauma, pregnancy and illness act as a stimulus for relapses.

1.5 LABORATORY DIAGNOSIS OF MALARIA

Irrefutable evidence of malaria is the demonstration of the parasite in stained blood films on microscopy by a competent and experienced person. An indirect evidence of malaria infection is made through serology (synonymous : immunodiagnosis), which has become of practical value in the recent years (1).

1.5.1 Microscopy

The definitive diagnosis of malaria has historically been based on the detection of parasites in the blood, and still today it supercedes all other laboratory diagnostic methods of malaria detection (14).

Two kinds of blood films, a thin film and a thick film could be made on glass slides of 25mm x 75 mm dimensions for detecting malaria parasites. Details of smear preparation are given in the Appendix.

Romanowsky in 1890 discovered an effective staining technique of plasmodia. This coupled with the aid of a modern high powered microscope leaves a competent microscopist to marvel at the accuracy with which the parasites could be visualised. Two types of stains being popularly used now in most laboratories are the Leishman and Giemsa stains. More details on instruction for preparation of stain used in this study can be found in the Materials and Methods section of this literature.

Having stressed the importance of blood film microscopy in the diagnosis of malaria, it has certain limitations which must be appreciated and include :

1. The difficulty in evaluating the significance of parasitemia in holoendemic regions where asymptomatic

parasitemia occurs in as many as 60-80% children and 20% adults.

2. Inappropriate self medication, which is a common practice in the developing countries or chemotherapy administered before and during the period when blood smear is made, suppresses parasitemia and reduces malaria parasites below detectable threshold of microscopy (15,16).

3. Inspite of active disease, microscopy shows negative results if blood films are made during the apyrexial interval, particularly in infections where P. falciparum matures synchronously and the parasites in the schizont stage disappear from the peripheral blood and sequester in the capillaries of the internal organs (15).

4. Negative results on microscopy can occur in all cases of primary infection during the first two or three days of the disease (15).

In the last two possibilities, serial blood films taken on intervals of 6 to 12 hours are required as long as diagnosis remains in doubt or until recovery supervenes.

1.5.2 Immunodiagnosis

Immunodiagnosis of malaria has received much attention during the recent years. The principle of immunodiagnosis is the detection of specific antimalarial antibodies. Since malarial antibodies are found in the sera of normal population in endemic areas, the test has to be quantitative when it is desired to distinguish between current, recent or past infections. However, measurement of antibody response has no part to play in the diagnosis of acute malaria, but progress may in the due course lead to immunological diagnosis of current infections (17).

Antibodies are first detected a few days after infection of the blood and antibody levels rise quickly to a plateau which is maintained for sometime after which a slow decline occurs (18,19,20). The exact time course of these events depends upon the species of the infecting malarial parasite and upon the previous experience of the host. In vivax malaria in which long term relapses can occur, antibody levels can fall between relapses but seldom disappear completely. However, after spontaneous resolution of the infection or following therapeutic cure, antibodies decrease rather rapidly and may become undetectable (17,21,22,23,24,25). Almost all the conventional serological methods have been tried at one time or another for assay of malarial antibodies, but the only techniques which have

found wider applications are agar-gel diffusion test, indirect haemagglutination test, indirect fluorescent antibody test, enzyme linked immunosorbent assay and radio-immunoassay.

Agar-Gel Diffusion Test

In this method the test sera are allowed to diffuse against soluble antigen in agar-gel. The presence and number of precipitin bands formed are an indication of the antibody contents of the test sera. The prevalence and number of precipitin bands increase with age, reflecting the exposure and development of immunity to malaria (15). The experience to date suggests that agar-gel diffusion is an excellent tool for the analysis of the multiple antigen-antibody responses in a community subjected to malaria. However owing to its rather low sensitivity and slowness it cannot be considered as being suitable for immunodiagnosis of malaria in the infected individual (17).

Indirect Haemagglutination (IHA) Test

This method is based on the agglutination of malarial antigen-coated carrier erythrocytes by malarial antibody. Crude soluble extracts of malarial parasites are used as the antigen and are fixed <u>in vitro</u> on to carrier sheep or human erythrocytes. The antigen employed popularly

for this purpose is derived from P. knowlesi since it can be readily available from the blood of the rhesus monkey, Macaca mulatta, after infecting it with P. knowlesi where it is capable of producing high parasitemia (26,27,28,29,30,31, 32). Dilutions of the test sera are then reacted with these sensitised erythrocytes. The pressure and amount of malarial antibody present are indicated by the serum dilution which leads to agglutinations. Negative results do occur as a result of non-specific agglutination factors which is a feature of some sera from tropical areas. It has also been reported that the test is rather insensitive at detecting antibodies in the early phases of a primary malaria infection, especially in young children which limits its usefulness (17).

Indirect Fluorescent Antibody (IFA) Test

The indirect method of immunofluorescence has been the preferred method of malarial antibody determination for the past decade (17,33,34). In this method the antigen consists of drops of infected blood dried on glass slides. These slides are reacted with dilutions of the test serum sample. After washing, the slides are reacted with a solution of fluorescein-labelled antiglobulin, and after another washing are examined on a fluorescent microscope. Antibodies in the test serum reacts with the malarial parasites in the blood smears and the fluorescein-labelled

antiglobulin then becomes fixed to the antigen-antibody complex. Antibody containing sera are indicated by fluorescence of the parasites in the slide antigen. The amount of antibody is shown by the last serial dilution that reacts and is given as the titre value for the sample concerned (17). Generally, fluorescence at a dilution of serum at 1:20 is regarded as the lowest positive titre and all dilutions over this titre showing fluorescence are indicated as a positive test for active malaria (1,26). Inspite of its limitations, indirect fluorescent antibody test has been widely and successfully used both for immunodiagnosis and seroepidemiology (21,26,35,36).

It has been confirmed that serological cross-reactivity occurs between P. falciparum and P. vivax (37). Since P. falciparum can be cultured in vitro (38), its antigens can be obtained in greater quantities than antigens of P. vivax for which no efficient culture technique is currently available. With the discovery of serological cross-reactivity between the two species, sera from P. falciparum infected patients can be cross-reacted with P. vivax antigen and vice versa (37).

Enzyme Linked Immunosorbent Assay (ELISA)

This test is analogous in concept to the IFA test. Soluble antigen from malaria infected blood is coated on to

a plastic surface which is reacted with the test serum sample. An enzyme labelled antiglobulin is then added, followed by a chromogenic enzyme substrate. The amount and rate of colour development are proportional to the amount of antibody in the test serum. The results can be read visually or with more accuracy on a photometer (17).

This technique is rather easy to perform, especially in the microplates and makes possible the processing of large number of samples. Its disadvantage lies in its inefficiency in detecting low antibody levels especially in children (39,40). ELISA has been successfully used in the serodiagnosis of vivax malaria (41).

Radioimmunoassay (RIA)

Radioimmonoassay can be performed in the same manner as ELISA, merely by substituting the enzyme label with I^{125} on the anti-immunoglobulin. In the initial studies the malarial antigen was fixed on to carrier erythrocytes but in the later studies plastic tubes or plates have been used. Results are comparable with ELISA, but the short life of the labelled reagents and the expensive equipment needed means that the test has little field value (17,42,43,44, 45).

1.5.3 Recent Advances in Laboratory Diagnosis of Malaria

Currently interest centres on the detection of circulating malarial antigen or parasite DNA as a diagnostic adjunct. The reliable detection of such circulating substances would be strong evidence of malaria even in the abscence of demonstratable peripheral blood parasitemia. As yet, however no test detects patent parasitemia more reliably than blood film examination and more sensitive methods are awaited, among which DNA probes hold considerable promise.

The development of specific DNA probes for identification of P. falciparum has been reported by various researchers (46,47). This method compared favourably in sensitivity with routine microscopy, detecting parasite densities as low as 40 parasites per µl of blood. However, these methods require organic extraction of the sample DNA from the patients blood, which is impractical under field conditions and when handling large numbers of samples. Recently, modifications of the conventional DNA probe method in malaria diagnosis has been reported in which blood samples are spotted onto filtration membranes directly after lysis and do not require additional sample manipulation. This method offers the advantage of a standardised procedure that can be used in a batchwise fashion on a large number of samples. Thus, a single technician can process thousands of

samples per day as compared to about 60 blood smears by ordinary microscopy, besides eliminating the individual reader bias (46).

Meanwhile, another advance that has been made in the laboratory diagnosis of malaria is the discovery of modified light microscopy by Kawamoto in 1991(48), who used fluorescence staining to detect malaria parasites in blood films, which is both sensitive, easier and less time consuming than ordinary Leishman or Giemsa staining. Fluorescence microscopy with a standard light microscope and a new interference filter which consists of a multi-layer excitation filter combined with barrier filter specially designed for the fluorescence stain, acridine orange, was used to detect malaria parasites in thin and thick blood films and rapid scanning of blood films was possible at a magnification of x 200 with the standardized lenses, indirectly. This may be a useful, economic system for rapid diagnosis of malaria. The interference filter was developed initially to look at fluorescein isothiocyanate (FITC) labelled immunofluorescence in the standard light microscope (49,50). This interference filter system designed for use with acridine orange in light microscope is useful, economic and easy to use for the laboratory diagnosis of malaria since inexperienced microscopists may have difficulty in reading blood smears, it is easier since artifacts and

pigment dots which are troublesome in ordinary stains cannot be seen.

Finally, Western Immunoblotting has also been used for detecting specific malarial antibodies of the IgG and IgM group, but the technique requires the use of expensive and sophisticated equipment and therefore impractical for routine diagnosis of malaria in the laboratory.

1.6 IMMUNITY TO MALARIA

The essential function of the immune system is defence against infection. Although the mechanisms of protective immunity against malaria are not fully understood, there is now ample evidence to indicate that humoral immune response (51), cell mediated immune responses and some non-specific factors are implicated in the potential of protective malaria immunity. Apart from these specific immune mechanisms, innate resistance of immunogenetic etiology is of significance in immunity to malaria. The various mechanisms of immunity to malaria have been explained briefly as follows:

1.6.1 Innate Immunity

An early finding in the immunity to malaria was that some individuals without previous experience of malaria were innately refractory to infection. Later, attention was

drawn to the singular resistance to infection by P. vivax that was possessed by Americans of Negroid descent. Such observations clearly established the evidence of important, non-immunological, possibly genetic mechanisms of resistance to malaria (51).

The main possible mechanisms of the innate immunity to malaria parasites have been envisaged :

1. The modification in the surface receptors of the erythrocytes to which the parasite adheres prior to penetration. For example, the Duffy blood group antigen (Fy^aFy^b) from the erythrocyte surface is refractory to the invasion by P. vivax, as found in the indigenous population of West Africa (1,51).

2. Other immuno-genetic factors which may exclude blockage of the surface receptors of erythrocytes but affect the intracellular development of plasmodia in the erythrocyte. These factors are listed as follows:

   (i) Modification in the molecular structure of haemoglobin, for example, in individuals possessing haemoglobin S (HbS) or sickle cell haemoglobin, the effects of P. falciparum are modified and the severe pathological lesions seldom occur, even in children. This accounts for

the high incidence of sickle cell anaemia in parts of Africa which are holoendemic for falciparum malaria. Erythrocytes with HbS show sickling in low oxygen conditions and has adverse effect on blood schizonts of P. falciparum, possibly as a result of the pressure exerted on the parasite in the sickle cell, although the exact mechanism of action is still unclear (1).

Foetal haemoglobin or haemoglobin F (HbF) is present in the infant at birth along with the normal haemoglobin in a ratio of 80% to 30% respectively. HbF is less suitable for the growth of P. falciparum, although its mechanism of action remains doubtful. HbF confers some protection, particularly in children with beta-thalassaemia during infancy and childhood.

(ii) Another genetic factor which can impede the intracellular development of parasites is the deficiency of glucose-6-phosphate-dehydrogenase (G-6-PD). The gene involved in the deficiency of this erythrocytic enzyme is present in various areas where malaria is endemic. It has been shown that malaria parasites in G-6-PD deficient cells are more easily damaged by effects of oxidants than the non-G-6-PD deficient cells (52).

1.6.2 Acquired Immunity

Acquired or adaptive immunity has two main features : memory and specificity and includes the involvement of both cellular and humoral responses which have been well established in malaria immunity (53). Acquired immunity to malaria is species, strain and stage specific (10,54). It is widely accepted that host responses to plasmodia are complex and involve a close collaboration between macrophages (non-specific components), T cells and B cells (54,61).

Humoral Immunity

This involves the action of immunoglobulins. During malarial infections, there is a marked increase in serum immunoglobulin levels, part of which is specific to malaria parasites. However, there is a poor correlation between the immune status of the host and the levels of antimalarial antibodies. Much of this antimalarial antibody is non-protective (55). There is evidence suggesting that antiplasmodial activity can be demonstrated in the IgG, IgM and IgA fractions of the immune human sera (56, 57) and IgG being the predominant group in established infections. The humoral immunity tends to operate by restricting

parasite replication only at the asexual erythrocytic level and does not block invasion of liver cells by sporozoites or totally inhibit the successful maturation of intrahepatic stages of plasmodia or gametocytogenesis (54).

Humoral immunity could be passive or active. Passive immunity is acquired by the newborns through transplacental transfer of maternal antimalarial IgG. These antibodies persist in the early months of life and decrease with time, usually declining completely within nine months. This accounts for the rare cases of malaria among newborns. With time the immunity to malaria slowly develops and years after continuous exposure to different species and strains of Plasmodium results in development of high antimalarial antibody titres among individuals in malaria endemic areas. It has been observed that children between ages of 6 months to 5 years are most susceptible to severe clinical disease. By adulthood the infected individuals show that little parasitemia may continue although the illness ends. It is also short lasting because immunity wanes without reinfection (55).

Antibodies however do not prevent infection but may prevent severe disease in the immune individuals which can be observed in mild clinical symptoms in relapses

after the primary attack of malaria. The inefficiency to kill malaria parasites completely through humoral involvement is probably due to the characteristic specific nature of antibodies, which are protective when the individual is challenged repeatedly by a homologous strain of the parasite, though some protection is also evident towards heterologous strain of the parasite (10).

Cellular Immunity

Cellular or cell mediated immunity involves the action of T cells on the parasites and is more effective in protection against malaria. Plasmodia being intracellular parasites evade the action of antibodies and complement and require to be destroyed through the action of T cells which react only with antigens associated with the hosts cell surface. Cell mediated immune responses and the pivotal role of T cells are implicated in potential of protective malaria immunity (58,59). In immunity to malaria T cells play a crucial role in regulating antibody production apart from giving rise to antibody-independent, non-specific cellular immunity (60).

1.6.3 Non-Specific Factors in Immunity to Malaria

This is the first line of action by the immune system and precedes the development of specific mechanisms of immunity. The non-specific factors include neutrophils and macrophages, both of which are phagocytic cells and act independently before any specific immune components are developed.

T cells are the key cells in inducing non-specific immunity. Their significant functions in immunity to malaria are listed as follows (54):

1. Attracting and activating macrophages which are able to destroy the intracellular malaria parasites through oxidative mechansisms following phagocytosis. Activated macrophages are also capable of producing a substance known as Tumour Necrosis Factor (TNF) which is capable of destroying malaria parasites.

2. Sensitized T cells are a source of interferon which has a potent inhibitory effect on the hepatic stages of the Plasmodium.

3. Interleukins are a soluble substance produced by the T cells or activated macrophages and show protective effect against malaria.

A significant finding in this context is that the epitopes recognized by human T cells reside within the conserved regions of the parasite which hampers the recognition of the immunogen by the T cell. This has important implication for designing an effective merozoite vaccine (62).

1.7 CONTROL

Control of malaria can be brought about by chemotherapy, chemoprophylaxis, use of insecticides, biological control, personal protection other than chemoprophylaxis and the introduction of antimalarial vaccines (63).

These are discussed below :

1.7.1 Drugs

The ideal antimalarial drug is effective against all forms of malaria, non-toxic, non-terratogenic and cheap. The major groups of drugs that are available are considered below :

Cinchona Alkaloids : Significant extracts of Cinchona bark are quinidine and quinine, both of which are highly active against the blood stages of all forms of plasmodia. Neither has activity against hepatic stages of the Plasmodium and, therefore, cannot be used for radical

treatment. Its toxicity prevents its use for chemoprophylaxis. However, it is used effectively for the treatment of chloroquine resistant plasmodia. These are the oldest non-synthetic antimalarials known (64).

Chloroquine It is a synthetic agent of the 4-amino-quinoline series. It is highly active against the asexual stages of plasmodia, but not active against hepatic stages and cannot be used for radical treatment. This drug can be used both for therapeutic and prophylactic purposes. Chloroquine was the ideal schizontocidal drug and used extensively until 1960's, when resistance against the drug by P. falciparum was first noticed. Over the period of time resistance to chloroquine by P. falciparum has spread rapidly and extensively. However, in areas where chloroquine resistance is not pronounced it is still the drug of choice against malaria (82). However, inspite of the emergence of chloroquine resistant strains of P. falciparum it is interesting to note that it is effective against all the other species of Plasmodium (64).

Amodiaquine : Like chloroquine, amodiaquine is a 4-aminoquinoline with the same mode of action and spectrum of activity. Due to its toxicity it should not be used for malaria prophylaxis. However, it remains in widespread use for treatment of chloroquine resistant infections (64).

Mefloquine : It is a quinoline methanol active against the blood-borne asexual stages of all species of human malaria parasites.

It is effective against certain chloroquine resistant and quinine resistant strains of P. falciparum. Unfortunately, resistance to mefloquine has already been reported, although the drug is not widely available. It is usually administered in combination with sulphadoxine and pyrimethamine (64).

Primaquine : This remains the only drug in current use which has activity against exoerythrocytic stages of infection and is thus able to achieve radical cure of vivax and ovale malaria, but it has no effect on the asexual blood forms of the parasite at therapeutically used doses (64).

Proguanil : The biguanides proguanil and its analogue chlorproguanil are active against the asexual blood stages of all species of human malaria, but the clinical response is slow and this limits the usefulness of the drug in the treatment of acute infections. Consequently, proguanil has been chiefly used for chemoprophylaxis. It is one of the safest antimalarials and it has seldom been implicated in serious toxicity (64).

Pyrimethamine : It is structurally related to the biguanides and its range of antimalarial activity is similar to the biguanides and used primarily for malaria

prophylaxis. Unlike biguanides, pyrimethmine has largely been used in combination with other drugs: with dapsone as Maloprim and with sulphadoxine as Fansidar. Occasional reports of pyrimethamine associated foetal deformity have been reported (64).

Dapsone : This is a diamino-diphenyl-sulphone and is schizontocidal for all forms of malaria, especially P. falciparum. Because of its slow onset of action its usefulness in the treatment of acute infections is limited and is principally been used in combination with pyrimethamine for malaria prophylaxis (64).

Sulphonamides : These are used regularly for the treatment of bacterial infections, but in combination with other agents they have useful antimalarial activity. Long acting sulfonamides such as sulfadoxine have been widely used as antimalarials for example in Fansidar. However, hypersensitivity is one of the well documented adverse effect of the drug (64).

Qinghaosu (Artemesinine) This sesquiterpene lactone derivative of the herb Artemisia annua is still under investigation, and clinical trials have yielded very promising results. It is a blood schizontocide against chloroquine sensitive as well as chloroquine resistant strains of P. falciparum (64).

Halofantrine : It is a phenanthrenemethanol and is an upcoming antimalarial still under investigation and not widely available for use. The drug is a potent blood schizontocide (1,64).

1.7.2 Vector Control

Vector control strategies for malaria include either killing the Anopheles mosquitoes or preventing them from biting the humans. The measures used to implement these strategies are listed below :

Use of insecticides : Adult mosquitoes can be killed by chemical insecticides like pyrethrum, chlorinated hydrocarbons, Dieldrin, Landrin, DDT etc., which are commercially available for spraying.

Biological control : The mosquito at the larval stage can be destroyed by introducing larvivorous fish into the mosquito breeding waters. Certain bacteria such as Bacillus thuringiensis, viruses, protozoa and nematodes can be lethally pathogenic to the vector.

Environmental measures : The control of anopheline breeding grounds is implemented in all urban and rural areas either by eliminating the mosquito breeding grounds or by the use of larvicidal oils on stagnant waters which contribute generously in the vector control for malaria (1,63).

Personal protection other than chemoprophylaxis: These include methods to prevent the mosquitoes from biting the humans, and can be implemented by various means such as use of mosquito nets. Recent inventions of a mosquito net impregnated by an insecticide has successfully reduced the incidence of malaria in China. Screening of houses reduces the access of mosquitoes in the dwellings. The use of mosquito coils can reduce the chance of mosquito bites in a closed environment. Finally, mosquito repellents can be applied to the skin, clothes and bed nets to prevent bites. Synthetic repellents like Indalone, Rutgers 612, Dimethyl pthalate are now commercially available (1,63).

1.7.3 Malaria Vaccines

In 1957, WHO aimed at malaria eradication. The overwhelming success of malaria control program, which used DDT to kill mosquitoes and chloroquine for chemotherapy, was so very impressive that scientific research on developing malaria vaccines was neglected (1,65).

However, unfortunately by the mid 70's the disease reappeared with a vengeance, largely because the malaria parasites had developed resistance against chloroquine, and also because the mosquito vector developed resistance to insecticides.

It is alarming that chloroquine and multiple resistant strains of P. falciparum are spreading in most of the tropical countries of the world and it is equally alarming that new antimalarial drugs, especially for the prophylaxis are not likely to become available in the near future (66), and that no new insecticides superior to DDT and economically usable by poor malaria-endemic countries are on hand. With these problems in malaria control, great hopes have been placed on the development of effective malaria vaccines to supplement existing and future malaria control measures.

Various methods of vaccination have been scrutinized and at present, there are three distinct malaria vaccines which are the focus of intense research.

(1) Sporozoite vaccine : This seems to block the entry of sporozoites into the hepatocytes. This would eliminate the initiation of infection. Sporozoites are highly immunogenic as proved by the high level of antisporozoite antibodies seen in the indigenes of endemic areas. X-ray irradiated sporozoites were used in the initial trials in human volunteers and showed small protection (67). Subsequently, the protein covering the surface of the sporozoite known as the circumsporozoite protein (CSP) was identified as a

protective immunogen (68,69,70). The genes coding for CS protein consists of a repetitive sequence of 4 amino acids, viz asparagine, alanine, asparagine and proline (NANP). Trials on sporozoite vaccine which consisted of various repetition numbers of NANP, adjuvanted in alum were carried out in 1987 and 1988 in human volunteers but did not yield satisfactory results. The problem mainly lies in the short duration of availability of sporozoites in the blood circulation of man, the time which does not suffice for specific antibody production and its action. The other problem lies in the species and strain variation which occurs and manifests in the antigenic variation in the CSP of the sporozoites and requires production of specific antibodies. The other problem lies in the species and strain specificity of the antisporozoite antibodies, and each fresh invasion by sporozoites needs a new compatible set of antibodies unless the infection is due to the invasion by homologous parasites, which is a rare condition in the endemic regions.

2. Merozoite vaccine : This aims to block the invasion of merozoites into the erythrocytes. The stage of asexual schizogony is responsible for the morbidity and mortality in the disease. A merozoite vaccine would prevent the disease or significantly alleviate its

course of infection. A number of blood stage antigens have been identified as possible vaccine candidates (71,72,73,74). The first clinical trial was carried out in Columbia (75), although limited immunogenicity and protection have been demonstrated. The results of the trials have been encouraging in the sense that the first generation of synthetic and recombinant malaria vaccines have been found to be safe in the humans. The dissatisfactory results of the merozoite-vaccine is because of poor immunological response and protection, which are due to the inherent biological nature of the immunogen, synthetic and recombinant proteins (62). Besides these problems, the natural immunity developed towards blood stage antigen can be extrapolated to the artificial immunity induced through vaccination. The immunity is usually species and stage specific. The antibodies are generally protective unless challenged with the homologous strains of the parasite.

3. Gametocyte vaccine : This is also known as the transmission-blocking vaccine; the vaccine is aimed to damage the sexual stages of the parasite life cycle. This vaccine would not prevent the infection or disease in the vaccine recepients but would block or alleviate the spread of the disease within the human population (62). Significant progress has been made in

identifying specific targets for antigamete immunity in a number of plasmodia species including P. falciparum and P. vivax (76). This target is a 25 kD protein and the gene encoding one of the target proteins has been cloned (77). One reason malariologists favour a gametocyte vaccine is that sexual stage of the parasite is likely to be far less immunologically variable than others. Many of the sexual stage proteins currently being engineered are expressed only when the parasite enters the mosquito and not noticed by the immune system, they are less likely to vary. Antibodies raised against them would be useful when the blood is carried into the mosquito where the gametocyte antigens are expressed. This is expected to block fertilization hence blocking the completion of the life cycle of the Plasmodium (78).

However, this vaccine is not without problems. Mendiz in 1987 (79) pointed out that low antigametocyte antibody titres in the mosquito blood meal may actually aid in bringing together the male and female gametocyte, increasing the chance of fertilization and making the disease more infectious.

So far no effective vaccine is available against malaria. There are various problems associated with the development of a proper malarial vaccine which include the

complex life cycle of the malaria parasite and changes in both morphology and antigen expression; and also because each developmental form of the parasite contains a myriad of distinct, stage specific antigens. Moreover, only a small proportion of these antigens are capable of stimulating the production of specific antibodies and are stage and species specific and keep reshuffling its structure on the molecular level to evade the host's immune responses.

The joint operation of T cells and immunoglobins are essential for the consequential immunity to malaria. Adjuvants are required to carry the immunogenic subunits of the parasite, the latter being too small to induce host immune response independently. The ideal adjuvant must be a potent inducer of both humoral and cell mediated immune responses and be safe for use in humans. Alum has been used as an adjuvant in the malaria vaccines and has been found safe and effective in inducing humoral immunity but deficient in inducing cell mediated immunity (80,81). The necessity for safe and effective adjuvants and for incorporation of T cell epitopes in the vaccine molecules are additional major constraints towards subunits malaria vaccine developments.

Designing a "cocktail" vaccine synthetically through recombined DNA technology, by combining a mixture of

sporozoites, merozoite and gametocyte defined antigens, each containing the conserved antigenic structures for eliciting T cell responses may circumvent some of the hurdles.

The sporozoite and merozoite vaccines are meant to eliminate the initiation of infection and to significantly modify the severity and course of infection, respectively. However, the antigens associated in both the stages are capable of changing its protein coat in ways offering a bewildering variety of identities that enable some sporozoites evade the hosts immune surveillance. Even a "cocktail" of immunogenic proteins is unlikely to be effective, because the parasite may quickly evolve new variants in its proteins.

Some malariologists still anticipate bright prospects for the development of effective malaria vaccines. Finally, if and when the malaria vaccine is available, it will serve as a supplement to the existing control measures.

1.8 AIMS AND OBJECTIVES

The present study was aimed to assess and re-evaluate the development of humoral immune responses during and after an attack of vivax malaria. There is no doubt that the major current role of malaria antibody testing is in the epidemiological studies where malaria is or was endemic. In this context serology can provide useful complimentary information to that given by conventional parasitological and clinical examination. The serological data can be considered as a combined indicator of point prevalence and recent period prevalence of the disease. The main practical aim of serology therefore is to first provide an index of prevalence of malaria in individuals living in malaria endemic areas prior to providing any assistance during and after malaria control or eradication schemes (17). Apart from assessing the development of humoral immunity during an attack of vivax malaria, an attempt was also made in this study to monitor such responses over a considerable period of time following an attack. The aim was to come out with some sort of a follow up study, in order to determine the rate at which malarial antibodies decline under field conditions; that is under normal circumstances when one is exposed to reinfections and other natural stresses.

The study was carried out in randomly selected patients in different age groups. Antibody monitoring was

carried out by means of indirect fluorescent antibody (IFA) test using fluorescein tagged antihuman antibodies. Some hand on information on the duration for which such malaria antibodies last was also considered necessary, for compiling a baseline data before starting any vaccination trials - if and when that may be. The study could also be significant in gaining some insight on the sequential appearance of circulating malaria antibodies during various phases of the disease, including post recovery period.

It would be quite in order to mention here that from a diagnostic point of view it is an established fact that malarial serology has little value in the diagnosis of acute malaria in the non-immune or semi-immune patients. However, estimation of antibody levels may have some diagnostic value in patients living in malarious areas with a history of fever but in whom the parasites have never been found due to the fact that blood films were taken at an inappropriate time, or after low-level antimalarial chemotherapy. In all such cases serology can be used to confirm or exclude malaria. It is especially important to detect P. vivax in such circumstances as this infection has often led to relapses and radical treatment is neccessitated (17).

The present investigations were therefore,

designed to study the P. vivax antibody profiles in individuals from three different age groups listed below :

1. Infants aged between 0 to 2 years with and without malaria.
2. Children aged between 2 to 14 years with and without malaria.
3. Adults aged between 15 to 56 years from both rural and urban areas with and without malaria.

A follow up study from sera samples from individuals 15 to 90 days after recovery from vivax malaria, and from relapse cases of vivax malaria was carried out in the study groups comprising of children (2-12 years) and adults (15-56 years).

MATERIALS AND METHODS

2.1 MATERIALS

Sera

Blood samples for sera separation were collected randomly from 63 inhabitants of both rural and urban areas in the Aligarh District of Uttar Pradesh (India).

The samples were collected from three different age groups. Within each sample group, the sera were collected from persons with : (1) active slide-proven vivax malaria; (2) clinically suspected malaria (negative slide test results); (3) repeated (or relapsing) vivax malaria, (4) healthy individuals, and (5) individuals 15 to 90 days after attack of vivax malaria. In infants aged between 0 to 2 years, samples were taken only from slide proven cases of vivax malaria, clinically suspected malaria cases and healthy controls.

Data on whether or not chemotherapy was administered along with other details were collected from each patient on a pre-designed proforma as given in the Appendix.

The various groups of subjects involved for sera collection are further categorized below :

AGE GROUPS/CASES	TOTAL SAMPLES
I. Infants Aged Between 0 to 2 Years	
1. Slide-Proven Vivax Malaria	4
2. Clinically Suspected Vivax Malaria (Smear Negative)	5
3. Healthy Controls	5
II. Children Aged Between 2 to 14 Years	
1. Slide-Proven Vivax Malaria	8
2. Clinically Suspected Vivax Malaria (Smear Negative)	4
3. 15 to 90 Days After Recovery From Vivax Malaria	3
4. Healthy Controls	4
5. Relapsing Vivax Malaria	3
III. Individuals Aged Between 15 to 56 Years	
1. Slide-Proven Vivax Malaria	8
2. Clinically Suspected Vivax Malaria (Smear Negative)	5
3. 15 to 90 Days After Recovery From Vivax Malaria	4
4. Healthy Controls	5
5. Relapsing Vivax Malaria	5

Antigen

P. vivax parasites from the infected blood slides

of vivax malaria patients, were used as antigen for IFA tests.

P. falciparum parasites were also used as antigen and were obtained from the in vitro cultures run in the laboratory.

Glassware, Syringes and Plastic Vials

The glassware used were from Pyrex and Company. Pre-sterilized, disposable plastic syringes and needles were obtained from Lycra (India) while plastic vials used for the storage of sera were bought from Basco Company (India).

Stain

Leishman stain was purchased in the powder form from BDH (England).

Fixative

Acetone used to fix the blood smears on the coverslips was obtained from Glaxo (India).

Chemicals

Dihydrogen Sodium Phosphate (monobasic), Disodium Hydrogen Phosphate (dibasic) and Sodium Chloride used for buffer preparation were obtained form BDH (India). Sodium Azide used for preservation was bought from Indian Drugs &

Pharmaceuticals Ltd. Methanol was obtained from Merck and Company Ltd.

Anticoagulant

Acid Citrate Dextrose was obtained from the Blood Bank, J.N. Medical College, A.M.U., Aligarh.

Medium

Waymouth's MB 752/1 medium was obtained from the Grand Island Biological Company (USA).

Conjugate

Fluorescein isothiocyanate (FITC) labelled anti-human IgG, IgM and IgA were obtained from Immunodiagnostics Ltd. (India).

Slides and Coverslips

Both slides and coverslips were obtained from Blue Star : Polar Industrial Corporation (India).

2.2 METHODS

2.2.1 Serum Collection

Between 3 to 5 ml of venous blood from the patients was collected in plain glass vials and left overnight in the refrigerator at 4°C and serum was separated

from the clot, subsequently. The serum was stored in 2 ml screw-capped plastic vials after labelling at -20°C till needed.

2.2.2 Antigen Preparation

P. falciparum antigen was prepared from infected red blood cells obtained from in vitro cultured parasites. In vitro cultures were made in the laboratory by fellow researchers as per modified method of Siddiqui (82).

P. vivax antigen was also used in this study. The parasites from the blood of slide-proven vivax malaria patients with high parasitemia (of about 0.8 to 1%) containing most parasites at the schizont stage were used as a source of antigen for serum antibody detection in IFA tests.

In some cases, venous blood from slide-proven vivax malaria patients with high parasitemia containing parasites at the trophozoite stage was collected in vials with anticoagulant. The infected blood samples were constituted to a 1:1 ratio with Waymouth's MB 752/1 medium ((83) and incubated at 37°C for 1-8 hours, while ordinary microscopy was done from time to time to check the development of initial trophozoite stages into mature trophozoites and schizonts. Parasites following their short term incubation

and maturity were eventually smeared on the coverslips at the appropriate time.

All smears were fixed in acetone for 5 to 10 minutes. The fixed coverslips were then wrapped in tissue paper and packed in airtight container in small batches and stored at -20°C till needed.

2.2.3 Microscopy

Ordinary light microscopy was performed on the blood smears of patients with primary patent vivax malaria, in order to further confirm the disease and determine the infecting species of the Plasmodium prior to serum collection and, of course, antigen preparation.

Leishman stain was used to stain the thin blood films. The detailed staining technique is given below:

Preparation of Leishman Stain

The stain used was in the form of powder. The strength of the solution used for staining was 0.15 per cent of the stain in methanol. An 0.15 gram sample of the stain was dissolved in 100 ml. of acetone-free pure methanol for obtaining the above stain concentration. The materials required for preparation were:

(i) ground-glass stoppered bottle of 150 ml. capacity

(ii) 100 ml. graduated glass cylinder

(iii) glass mortar and pestle

All the articles were thoroughly cleared and rinsed with methanol before use. A 100 ml. methanol was first measured in the graduated measuring cylinder. The weighed amount (0.15 g) of Leishman powder was placed in the mortar and ground into a paste by adding methanol in small quantities (about 2 ml. each time). The dissolved stain was carefully decanted off from time to time into a glass-stoppered bottle. The individual stain was grinded again with methanol till no residue was left and the whole methanol had been used up. The stoppered-glass bottle with the stain was kept in an incubator at 37°C for 24 hours while being filtered after which time it was ready for use (11).

Staining Procedure

(i) Leishman's stain was poured from a drop bottle by means of pipette over the dried film and allowed to remain for 30 seconds.

(ii) The stain was diluted with twice its volume of distilled water which was enough to be neutralised, or left it slightly alkaline (pH 7.0 to 7.2). It was later covered to prevent it from drying.

(iii) The diluted stain was allowed to remain on the slide for 10 to 15 minutes.

(iv) The slide was held under an open tap and the stain flushed by gentle flow of water. The reverse side of the slide was cleared by rubbing it well with wet, squeezed cotton wool.

(v) The slide was kept in an upright position (film-slide inwards) to drain and dry.

(vi) The dried stained film was examined in the oil immersion objective of the microscope.

2.2.4 Buffer Preparation

0.1 M Phosphate Buffered Saline (PBS) at pH 7.2 was the buffer used in this study. Details of its preparation is as follow :

Phosphate buffered saline was prepared by incorporating Dihydrogen Sodium Phosphate, Disodium Hydrogen Phosphate and Sodium Chloride. The solution was made at 0.01 molarity, pH 7.2.

The weight of the chemicals involved were calculated by substitution in the following formula;

$$\text{weight (g)} = \frac{\text{molarity} \times \text{molecular weight} \times \text{volume (ml.)}}{1000}$$

Thus, the weight of the components in 1000 ml solution was calculated as:

1. Dihydrogen Sodium Phosphate (monobasic):

$$\text{weight (g)} = \frac{0.01 \times 156.01 \times 1000}{1000} = 1.56 \text{ g}$$

2. Dihydrogen Hydrogen Phosphate (dibasic):

$$\text{weight (g)} = \frac{0.01 \times 178.16 \times 1000}{1000} = 1.78 \text{ g}$$

3. Sodium Chloride :

$$\text{weight (g)} = \frac{0.15 \times 58.45 \times 1000}{1000} = 8.77 \text{ g}$$

The final volume was made to 1000 ml in double distilled water and the pH adjusted to 7.2. A pinch of Sodium Azide was added to the buffer for preservation and the buffer stored at 4°C in the refrigerator until needed.

Indirect Fluorescent Antibody (IFA) Test Technique

The procedure outlined in the World Health Organization Memorandum (1974) was used with a few modifications (84).

Antisera and antigen coverslips were taken out from storage, and raised to room temperature for 20 to 30 minutes. Antigen coverslips were washed for 10 minutes in distilled water by slowly agitaing them in a Petri dish containing distilled water. After the coverslips were washed, they were removed from the bath, and the excess fluid was removed by careful blotting. The antigen coverslips were labelled and placed in moist chambers prepared by lining Petri dishes with wet filter paper.

Antisera to be tested were diluted with PBS (pH 7.2) in two-fold steps, starting at a dilution of 1:10. About 0.05 ml. of each dilution was dispensed on labelled antigen smears. The chamber was covered and incubated at 37°C for 30 minutes.

After incubation, excess serum was removed from each coverslip with a gentle stream of PBS followed by gently agitating the coverslip in a Petri dish containing PBS for 5 minutes, discarding and replacing it with fresh PBS to complete a series of 3 consecutive washings. The coverslips were then removed from the wash and replaced in the moist chamber.

The fluorescein isothiocyanate conjugated (FITC) antihuman IgG, FITC antihuman IgM and FITC antihuman IgA diluted to an optimal strength in PBS were applied to

antigen areas on individual coverslips with the same serum dilution and allowed to react for 20 to 30 minutes at 37°C. Excess conjugate was removed by a gentle saline stream followed by three serial washings in PBS of 5 minutes each, and then blotted dry with filter paper. The slides were then mounted with the smeared coverslips using buffered glycerol. The edges of the coverslips were sealed with finger nail varnish.

Two controls, a negative control made by placing a drop of PBS on one slide and a positive control made by placing a drop of a known positive antimalarial serum on the other were also made for examination and comparison.

The stained coverslips were examined immediately under the fluorescent microscope (Olympus Co. Ltd., Japan) equipped with Exciter Filter UGI and Barrier Filter L420, and a D.C. 200-watt high pressure mercury burner employed as the light source. The last serum dilution yielding detectable fluorescence was considered as the end-point and the titre was expressed as a reciprocal of that dilution i.e 1:20, 1:40 and so on.

RESULTS

Antigen Preparation

Continuous *in vitro* cultures of *P. falciparum* carried out in our laboratories by fellow researchers were used for obtaining the antigen for indirect fluorescent antibody (IFA) test (2). The *in vitro* cultures attained a peak parasitemia level of 10% after the seventh week, yielding a population of parasites consistng mainly of ring forms and trophozoites. Schizonts were found to constitute only 10-25% of the population. Gametocytes were only rarely observed. Such a parasite yield was optimum for preparing antigen smears on coverslips that could serve to carry out the IFA tests.

P. vivax antigen was either obtained directly from infected patients with most parasites in the mature trophozoite and schizont stages and having a parasitemia level ranging between 0.8-1%. Alternatively, the antigen used was prepared from *P. vivax* infected patients' blood with similar parasitemic levels. Few samples of infected blood with early erythrocytic stages of *P. vivax* were incubated for short term cultures in vials containing Waymouth's MB 752/1 medium and anticoagulant(Acid Citrate Dextrose) at 37°C. Such samples were found to yield a parasite population with a majority of the immature stages having developed into late trophozoite and schizont stages

within 4 to 8 hours. Thin blood smears suitable for the IFA tests were made, subsequently.

Indirect Fluorescent Antibody (IFA) Test

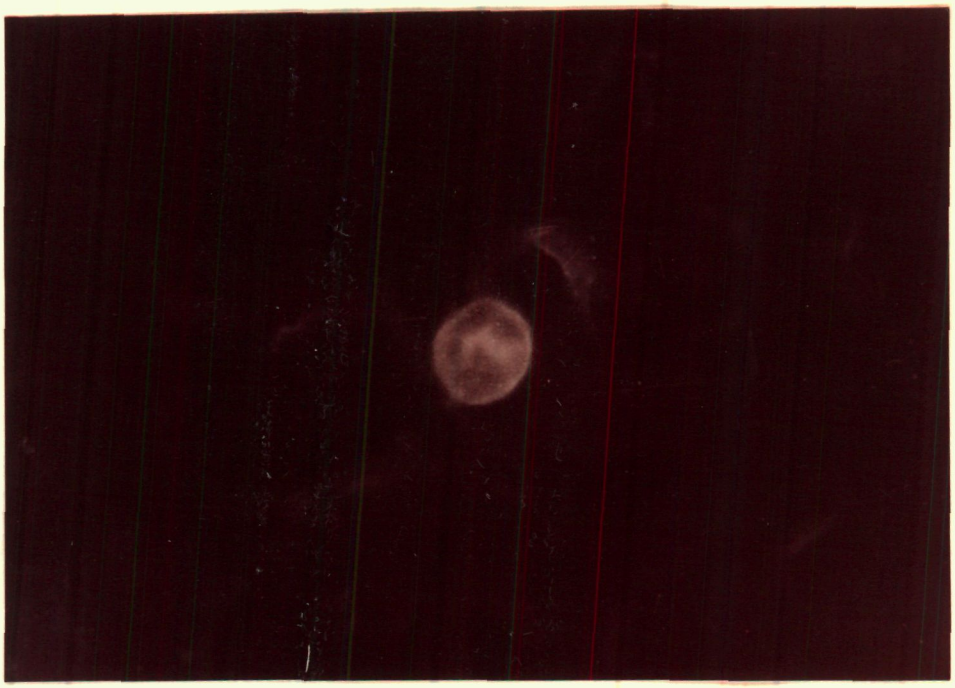
The IFA test was performed according to a modified procedure outlined by the World Health Organization Memorandum (87). The working dilution of the fluorescein isothiocyanate conjugated (FITC) antihuman IgG, IgM and IgA was determined and a dilution of 1:20 was found optimal for use in the IFA test. Immune sera samples and acetone-fixed antigen smears on coverslips when reacted with FITC antihuman antibodies produced a greenish fluorescence under ultravoilet light. An illustration of this is presented in Figure 1.

Control slides smeared with non-immune serum did not show any fluorescence, while the positive control slides made by using a known positive antimalarial serum in the IFA tests produced detectable fluorescence.

Tables I(1), I(2) and I(3) show IFA test results in infants aged between 0 to 2 years with slide-proven vivax malaria, clinically suspected vivax malaria and in healthy controls, respectively.

All sera obtained from infants (0-2 years) with slide-proven vivax malaria produced fluorescence at

Figure 1: Fluorescence Produced in the Indirect Fluorescent Antibody (IFA) Test on Acetone - Fixed Blood Film Infected with *P. vivax* (x 1000)



dilutions of 1:10 and 1:20 when reacted with FITC antihuman IgG, indicating the presence of malaria specific IgG in the test sera (Table I(1)). However only 25% test sera produced fluorescence at a dilution of 1:40. At the next consecutive dilutions of 1:80 and 1:160 no fluorescence with FITC antihuman IgG was detected in any test serum. Only 50% test sera produced fluorescence, thus indicating the presence of malaria specific IgM at a dilution of 1:10 and 1:20 when reacted with FITC antihuman IgM, while only 25% samples gave positive results for the presence of malaria specific IgM at a serum dilution of 1:40. No fluorescence was detected in dilutions of 1:80 and above. IgA against malaria was also not detected in any serum sample from the infants' (0-2 years) group having a slide-proven vivax malaria.

IFA antibody titres recorded in infants (0-2 years) with clinically suspected vivax malaria, having negative results of peripheral blood smear microscopy are provided in Table I(2). A total of five sera samples were studied in this group. Malaria specific IgG was detected in 40% samples at a dilution value of 1:10, reducing to 20% at a serum dilution of 1:20. No antibodies were detected at the higher dilutions of 1:40, 1:80 and 1:160. Malaria specific IgM was detected in 80% sera samples at a dilution of 1:10 and decreased to 60% at a serum dilution of 1:20, and further dropped to only 20% at a serum dilution of 1:40.

No malaria specific IgM was detected in serum dilution of 1:80 and above. IgA against malaria was detected in 20% test sera at a dilution of 1:10; higher dilutions of 1:20 and 1:40 yielded negative results for the presence of malaria specific IgA.

Healthy controls in the infants' (0-2 years) group were also tested for the presence of malarial antibodies using the IFA test. Results of the study are indicated in Table I(3). Highest level of antimalarial IgG was observed in 60% healthy infants at a serum dilution of 1:10. The levels decreased in the higher dilutions of the test sera with only 20% healthy infants showing the presence of specific IgG for malaria at 1:20 and 1:40 serum dilutions. No IgG against malaria was detected when sera were diluted to 1:80 and 1:160.

Antimalarial IgM was detected in 60% of the tested healthy infants at a serum dilution of 1:10. At a further serum dilution of 1:20 only 20% healthy infants sera tested were found to have malaria specific IgM. All other higher dilutions of 1:40, 1:80 and 1:160 gave negative results. Malaria specific IgA could not be detected in any test sera of this group.

Table I (1)

IFA TEST TITRES IN INFANTS AGED BETWEEN 0 TO 2 YEARS
(Slide-Proven Vivax Malaria Cases)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 4	1:10	4 (100%)	2 (50%)	0 (0%)
	1:20	4 (100%)	2 (50%)	0 (0%)
	1:40	1 (25%)	1 (25%)	-
	1:80	0 (0%)	0 (0%)	-
	1:160	0 (0%)	0 (0%)	-

Table I (2)

IFA TEST TITRES IN INFANTS AGED BETWEEN 0 TO 2 YEARS
(Clinically Suspected for Malaria; Slide Negative Cases)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 5	1:10	2 (40%)	4 (80%)	1 (20%)
	1:20	1 (20%)	3 (60%)	0 (0%)
	1:40	0 (0%)	1 (20%)	0 (0%)
	1:80	0 (0%)	0 (0%)	-
	1:160	0 (0%)	0 (0%)	-

* - Number of Samples (n)

Table I (3)

IFA TEST TITRES IN INFANTS AGED BETWEEN 0 TO 2 YEARS
(Healthy Controls)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 5	1:10	3 (60%)	3 (60%)	0 (0%)
	1:20	1 (20%)	1 (20%)	0 (0%)
	1:40	1 (20%)	0 (0%)	0 (0%)
	1:80	0 (0%)	0 (0%)	-
	1:160	0 (0%)	0 (0%)	-

* - Number of Samples (n)

IFA test results in children aged between 2-14 years are provided in Tables II(1), II(2), II(3), II(4) and II(5). Children (2-14 years) with slide-proven vivax malaria were found to have higher levels of antimalarial IgM as compared to antimalarial IgG (Table II(1)). One hundred percent samples were found positive for the presence of antimalarial IgM in all serum dilutions of 1:10, 1:20, 1:40, 1:80 and 1:160, while malaria specific IgG was found in all sera at 1:10 and 1:20 dilutions only. Subsequent dilutions of 1:40 and 1:80 showed 62.5% sample positivity indicating for presence of IgG. At a further at a dilution of 1:160 about 25% sera indicated the presence of specific IgG dropping down to only 12.5% in sera samples at dilutions of 1:320 and 1:640. No IgG was detectable at a dilution of 1:1280.

IgM titres in this group of children with slide-proven vivax malaria fell from 100% samples showing the presence of antimalarial IgM at dilutions upto 1:160 to only 87.5% sample positivity at a serum dilution of 1:320. This further declined to only 25% sample positivity for the presence of IgM at a dilution of 1:640. Further serum dilutions gave negative results.

Malaria specific IgA was detected in 12.5% test samples at a dilution of 1:10. Further dilutions gave

negative results for IgA.

Children with clinical symptoms of vivax malaria but in whom the blood smear microscopy detected no malaria parasites were found to have malaria specific IgG in all the four sera samples studied at a dilution of 1:10 (Table II(2)). At a serum dilution of 1:20, the percentage sample positivity dropped to only 25%. The sample positivity rose unexpectedly to 50% at the next higher serum dilutions of 1:40 and 1:80; this was probably due to some experimental error. No IgG was detected in serum dilutions above 1:80.

IgM in this group of children (Table II(2)) was found in all cases (100%) at a serum dilution of 1:10, and at a further dilution of 1:20 and 1:40, only 75% test sera indicated the presence of specific IgM, declining to only 50% at a dilution of 1:80. Only 25% samples showed the presence of IgM by the IFA test at a dilution of 1:160 and further dilutions of 1:320 and 1:640 produced negative results.

A quarter portion of sera of children with clinically suspected malaria had indicated the presence of malaria specific IgA at dilutions of 1:10 and 1:20, further dilutions of 1:40, 1:80 and 1:160 gave negative results.

Table II(3) summarizes the IFA test results on three sera samples from children (2-14 years) obtained 15 to

90 days after recovery from a confirmed attack of vivax malaria. IgG levels were highest as compared to the levels of IgM and IgA showing a positivity in 100% test sera in all dilutions of 1:10, 1:20, 1:40. 1:80, 1:160 and 1:320. Later, it fell to 66.6% sample positivity at a serum dilution of 1:640 and declined further to only 33.3% samples showing the presence of malaria specific IgG at a serum dilution of 1:1280.

Presence of IgM was indicated in 100% test sera at dilutions of 1:10 and 1:20. These values later declined to 66.6% sample positivity at a dilution of 1:40 and only 33.3% sera were positive at 1:80 dilution. No antibody was detectable in further dilutions of 1:160 and 1:1280. IgA against malaria was not found in any test sera.

Table II(4) shows the IFA test results in sera samples of apparently healthy children (2-14 years). Only 50% cases were found to have malaria specific IgG at a dilution of 1:10, thereafter further dilutions gave negative results for the presence of malaria specific IgG. On the other hand, IgM levels were comparatively higher with 100% samples showing fluorescence at dilution of 1:10 and 1:20. Only 25% test sera showed fluorescence at 1:40, 1:80 and 1:160 dilutions. No fluorescence was recorded in serum dilution of above 1:160. IgA was found in low amounts in

only 25% test sera at a dilution of 1:10 and 1:20, subsequent higher dilutions gave negative results.

Relapses due to P. vivax infections lead to a rapid rise in the IgG titres (42). A total of three sera samples from children (2-14 years) having slide positive vivax malaria and presenting with a history of previous attacks of vivax malaria 3 to 6 months before the prevailing P. vivax infection were tested for antimalarial antibody levels using the IFA test. The results are summarized in Table II(5). All the three test sera showed fluorescence at a dilution of 1:10 and 1:20 when reacted with FITC antihuman IgG. At dilutions of 1:40, 1:80, 1:160, 1:320 and 1:640 only one out of the three (33.3%) test sera gave positive results. The next higher dilution of 1:1280 gave negative results.

IgM was detected more significantly in all the test sera giving a positive result for the presence of antimalarial IgM at serum dilutions of 1:10 and 1:20. Two out of the three test sera indicated IgM antibodies in dilutions of 1:40, 1:80 and 1:160. Negative results were recorded in dilution of 1:320 and above for the presence of malaria specific IgM.

No samples in this group were tested for the presence of antimalarial IgA.

Table II (1)

IFA TEST TITRES IN CHILDREN AGED BETWEEN 2 TO 14 YEARS
(Slide-Proven Vivax Malaria Cases)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 8	1:10	8 (100%)	8 (100%)	1 (12.5%)
	1:20	8 (100%)	8 (100%)	0 (0%)
	1:40	5 (62.5%)	8 (100%)	0 (0%)
	1:80	5 (62.5%)	8 (100%)	0 (0%)
	1:160	2 (25%)	8 (100%)	0 (0%)
	1:320	1 (12.5%)	7 (87.5%)	-
	1:640	0 (0%)	2 (25%)	-
	1:1280	0 (0%)	0 (0%)	-

Table II (2)

IFA TEST TITRES IN CHILDREN AGED BETWEEN 2 TO 14 YEARS
(Clinically Suspected for Malaria; Slide Negative Cases)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 4	1:10	4 (100%)	4 (100%)	1 (25%)
	1:20	1 (25%)	3 (75%)	1 (25%)
	1:40	2 (50%)	3 (75%)	0 (0%)
	1:80	2 (50%)	2 (50%)	0 (0%)
	1:160	0 (0%)	1 (25%)	0 (0%)
	1:320	0 (0%)	0 (0%)	-
	1:640	-	0 (0%)	-
	1:1280	-	-	-

* - Number of Samples (*n*)

Table II (3)

IFA TEST TITRES IN CHILDREN AGED BETWEEN 2 TO 14 YEARS
(Post Recovery Cases)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 3	1:10	3 (100%)	3 (100%)	0 (0%)
	1:20	3 (100%)	3 (100%)	0 (0%)
	1:40	3 (100%)	2 (66.6%)	0 (0%)
	1:80	3 (100%)	1 (33.3%)	-
	1:160	3 (100%)	0 (0%)	-
	1:320	3 (100%)	0 (0%)	-
	1:640	2 (66.6%)	0 (0%)	-
	1:1280	1 (33.3%)	0 (0%)	-

Table II (4)

IFA TEST TITRES IN CHILDREN AGED BETWEEN 2 TO 14 YEARS
(Healthy Controls)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 4	1:10	2 (50%)	4 (100%)	1 (25%)
	1:20	0 (0%)	4 (100%)	1 (25%)
	1:40	0 (0%)	1 (25%)	0 (0%)
	1:80	0 (0%)	1 (25%)	0 (0%)
	1:160	-	1 (25%)	0 (0%)
	1:320	-	0 (0%)	-
	1:640	-	0 (0%)	-
	1:1280	-	0 (0%)	-

* - Number of Samples (n)

Table II (5)

IFA TEST TITRES IN CHILDREN AGED BETWEEN 2 TO 14 YEARS
(Relapse/Reinfection Cases)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 3	1:10	3 (100%)	3 (100%)	-
	1:20	3 (100%)	3 (100%)	-
	1:40	1 (33.3%)	2 (66.6%)	-
	1:80	1 (33.3%)	2 (66.6%)	-
	1:160	1 (33.3%)	2 (66.6%)	-
	1:320	1 (33.3%)	0 (0%)	-
	1:640	1 (33.3%)	0 (0%)	-
	1:1280	0 (0%)	0 (0%)	-

* - Number of Samples (n)

Sera from adults aged between 15 to 56 years were tested, the results of which are provided in Tables III(1) to III(5).

A total of eight adults (15-56 years) with slide-proven vivax malaria volunteered to donate blood for IFA studies. In such IFA tests (Table III (1)), all the samples showed the presence of IgG when sera were diluted to titres of 1:10, 1:20 and 1:40. Six out of the eight samples (75%) gave positive results for antimalarial IgG when diluted to 1:80, while only half of the samples indicated the presence of IgG at 1:160 dilution. At a serum dilution of 1:320, only 25% samples showed the presence of malaria specific IgG. In dilutions of 1:640 and above no malaria specific IgG was detected.

IgM was demonstrated in all the eight sera samples at dilutions of 1:10 to 1:80. About 25% samples showed antimalarial IgM antibodies at a dilution of 1:160, while at dilutions of 1:320 and 1;640 only one out of eight samples showed fluorescence. No fluorescence was recorded at a next higher dilution of 1:1280, indicating the absence of antimalarial IgM in the test sera.

IgA was present in 25% samples at dilutions of 1:10 and 1:20. No IgA was detected at higher sera dilutions of 1:40, 1:80 and 1:160.

Sera from adults in whom malaria was diagnosed on the basis of clinical symptoms (slide tests detected no parasites in the peripheral blood) were obtained for IFA test. A total of 5 samples were tested. The results of the test are shown in Table III(2).

IFA test detected malaria specific IgG in all the five samples at 1:10 dilution. Further dilutions of 1:20, 1:40 and 1:80 were found positive for IgG in only 40% samples. At the next higher serum dilution of 1:160 only 20% samples produced fluorescence. No fluorescence was seen in dilutions of 1:320, 1;640 and 1:1280 indicating the absence of malaria specific IgG at these dilutions.

IgM was detected in all sera diluted to 1:10 and 1:20, the levels declined as indicated at a dilution of 1:40 and 1:80 where only 60% samples produced fluorescence. At dilutions 1:160 and 1:320 the antimalarial IgM antibodies were detectable in only 20% samples. Further dilutions yielded negative results.

Antimalarial IgA was detected only at a dilution of 1:10, in 20% sera samples, subsequently higher dilutions of the test sera gave negative results.

IFA studies were also carried out on 4 sera from adults obtained 30 to 90 days after the recovery from an

attack of slide-proven malaria (Table III(3)). High antimalarial IgG levels were found in this group of adults with 100% samples indicating the presence of malaria specific IgG in all dilutions starting from 1:10 to 1:160. The last serum dilution indicating the presence of IgG was 1:640.

IgM levels were comparatively low with only 1:10 and 1:20 dilutions giving positive results in 100% samples. At a dilution of 1:40 and 1:80 only 25% samples remained positive for the presence of IgM. Subsequent dilutions of 1:160, 1:320 and 1:640 yielded negative results. No samples were run for the detection of malaria specific IgA.

Sera from healthy adults (15-56 years) used as controls showed 80% sample positivity at a dilution of 1:10 and 1:20. But only one out of a total of five sera samples (20%) produced fluorescence at subsequent dilutions, reaching an end-point at a dilution of 1:160 (Table III(4)).

Compared to 100% positive sera at 1:10 and 1:20 dilutions IgM values were somewhat lower to only 40% positive samples at 1:40 and 1:80 dilutions in the healthy control group of adults. No fluorescence was found in the higher dilutions at 1:160 and 1:320.

IgA was found in 40% samples from the healthy adults' group at 1:10 dilutions and in only 20% samples at

1:20 dilutions. No fluorescence was found at dilutions of 1:40 and 1:80.

Sera from relapse cases in adults were taken on the same criterion as for the relapse patients in 2 to 14 years age group.

Specific IgG against Plasmodium was detectable in all the five test sera at dilutions of 1:10 and 1:20 (Table III(5)). At dilutions of 1:40, 1:80 and 1:160, 80% samples indicated the presence of antimalarial IgG, declining to only 50% positive samples at a serum dilution of 1:320. No antimalarial IgG was detected at a dilution of 1:640. At a higher dilution of 1:1280. Only one sample out of the five test sera produced fluorescence.

Antimalarial IgM was found in all the five test sera at dilutions of 1:10, 1:20 and 1:40, dropping sharply to only one test serum yielding fluorescence at a dilution of 1:80. Subsequent higher dilutions of 1:160, 1:320 and 1:640 yielded negative results for the presence of malaria specific IgM.

IgA against malaria was not tested in any sera sample of this group.

Table III (1)

IFA TEST TITRES IN ADULTS AGED BETWEEN 15 TO 56 YEARS
(Slide-Proven Vivax Malaria Cases)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 8	1:10	8 (100%)	8 (100%)	2 (25%)
	1:20	8 (100%)	8 (100%)	2 (25%)
	1:40	8 (100%)	8 (100%)	0 (0%)
	1:80	6 (75%)	8 (100%)	0 (0%)
	1:160	4 (50%)	2 (25%)	0 (0%)
	1:320	2 (25%)	1 (12.5%)	-
	1:640	0 (0%)	1 (12.5%)	-
	1:1280	0 (0%)	0 (0%)	-

Table III (2)

IFA TEST TITRES IN ADULTS AGED BETWEEN 15 TO 56 YEARS
(Clinically Suspected for Malaria; Slide Negative Cases)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 5	1:10	5 (100%)	5 (100%)	1 (20%)
	1:20	2 (40%)	5 (100%)	0 (0%)
	1:40	2 (40%)	3 (60%)	0 (0%)
	1:80	2 (40%)	3 (60%)	0 (0%)
	1:160	1 (20%)	1 (20%)	0 (0%)
	1:320	0 (0%)	1 (20%)	-
	1:640	0 (0%)	0 (0%)	-
	1:1280	0 (0%)	0 (0%)	-

* - Number of Samples (n)

Table III (3)

IFA TEST TITRES IN ADULTS AGED BETWEEN 15 TO 56 YEARS
(Post Recovery Cases)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 4	1:10	4 (100%)	4 (100%)	-
	1:20	4 (100%)	4 (100%)	-
	1:40	4 (100%)	1 (25%)	-
	1:80	4 (100%)	1 (25%)	-
	1:160	4 (100%)	0 (0%)	-
	1:320	1 (25%)	0 (0%)	-
	1:640	2 (50%)	0 (0%)	-
	1:1280	0 (0%)	-	-

Table III (4)

IFA TEST TITRES IN ADULTS AGED BETWEEN 15 TO 56 YEARS
(Healthy Controls)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
* n = 5	1:10	4 (80%)	5 (100%)	2 (40%)
	1:20	4 (80%)	5 (100%)	1 (20%)
	1:40	1 (20%)	2 (40%)	0 (0%)
	1:80	1 (20%)	2 (40%)	0 (0%)
	1:160	1 (20%)	0 (0%)	-
	1:320	0 (0%)	0 (0%)	-
	1:640	0 (0%)	-	-
	1:1280	-	-	-

* - Number of Samples (n)

Table III (5)

IFA TEST TITRES IN ADULTS AGED BETWEEN 15 TO 56 YEARS
(Relapse/Reinfection Cases)

	IFA test titres	Serum Antibodies in IFA		
		IgG	IgM	IgA
*n = 5	1:10	5 (100%)	5 (100%)	-
	1:20	5 (100%)	5 (100%)	-
	1:40	4 (80%)	5 (100%)	-
	1:80	4 (80%)	1 (20%)	-
	1:160	4 (80%)	0 (0%)	-
	1:320	2 (40%)	0 (0%)	-
	1:640	0 (0%)	0 (0%)	-
	1:1280	1 (20%)	-	-

* - Number of Samples (n)

Sera samples were also collected from six patients ranging from 2 1/2 to 5 years of age in whom P. vivax was reported on smear microscopy but were found slide negative after antimalarial chemotherapy. In these cases, the results on serum dilutions which reacted with FITC antihuman IgG and IgM to produce detectable fluorescence are shown in Table IV for each of the 6 patients.

In all the cases, the end serum titre indicating the presence of IgM was always higher compared to the last dilution at which IgG was detectable. For example, in one patient aged 2 1/2 years following chemotherapy the antimalarial IgM was detectable at a dilution upto 1:320, while IgG was detectable only upto 1:40 dilution. These results are further illustrated in Figure 2.

Table IV

Antimalarial Antibody Profiles in Slide-Proven Vivax Malaria Cases After Administration of Chemotherapy

Age (Years)	IgG	IgM
2 1/2	1:40	1:320
24	1:80	1:160
27	1:40	1:160
50	1:80	1:320
8	1:40	1:160
14	1:80	1:160

Figure 2: Profiles of malaria specific IgG & IgM levels in 6 vivax malaria cases after treatment

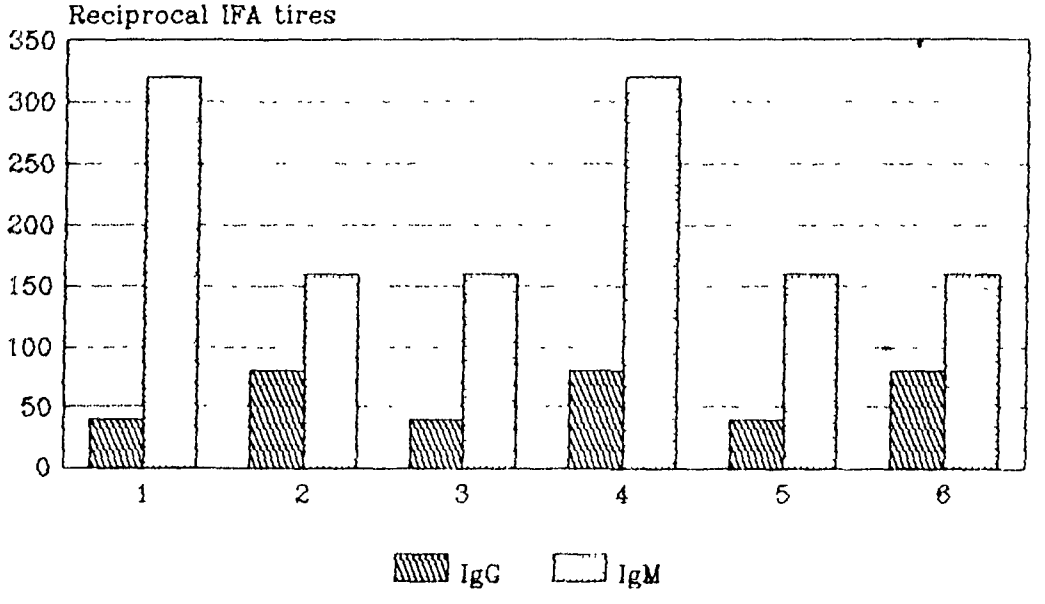
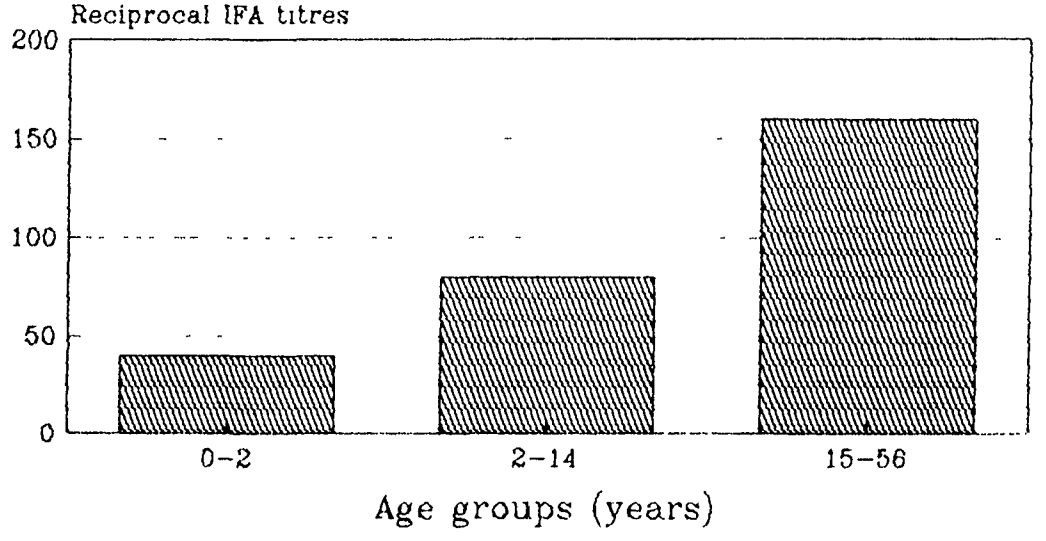


Figure 3: Profiles of malaria specific IgG titres in healthy individuals of Aligarh District increasing with age.



DISCUSSION AND CONCLUSION

4.1 DISCUSSION

Repeated malaria infection in individuals living in malaria endemic and particularly in holoendemic areas results in the development of immunity against pathogenic asexual stages of Plasmodium parasites; such immunity reduces parasite load and severity of the clinical illness. During malaria infection, there is a marked increase in serum immunoglobulin levels, part of which is specific to malaria parasites. It has already been established that specific malarial antibody activity can be demonstrated in IgG, IgM and IgA fractions of the immune sera (56,57) and IgG is predominant in established infections (53).

In order to investigate the natural response to erythrocytic asexual stage antigens, the present study sought to test sera samples for the presence of malaria specific IgG and IgM antibodies (in a few cases malaria specific IgA was also screened) in different age groups ranging from 0 to 56 years in the inhabitants of Aligarh District, which is known to be endemic for malaria (85).

The method employed for the antibody detection in this investigation was the indirect fluorescent antibody (IFA) test. IFA test has been used extensively and at the present time it is considered as the test of choice for determination of malarial antibody (17,34). IFA test detects

antibody very soon after the blood is invaded and the percentage of false negatives is lower than with other tests. This may be due to the fact that whole parasite is used as an antigen in IFA test (42).

Results from this study indicate that people repeatedly exposed to natural infection by P. vivax in the Aligarh District show the presence of malaria specific antibodies of the IgG and IgM fractions.(In some cases IgA was also found on screening).

Infants between 0-2 years of age were found to have low levels of IgG against Plasmodium, irrespective of whether they had a positive slide test or not.

It was found that a cross-sectional serological survey could provide an index of the overall level of malarial experience by a community (86,87,88). The results of these studies were in agreement showing that new-born infants had high levels of placentally-transmitted antibody which declined over the first year of life and then, following malarial exposure, antibody levels increased over a period of time (89).

The maternally acquired antibodies belong to the IgG class only (90). In this study, however, IgM was also detected along with IgG, suggesting exposure to natural

infection and the initial development of humoral immunity. The presence of IgM therefore, does not confirm the presence of IgG in the infants of this area to maternal origin. Development of humoral immunity to malaria was found to be age related. The reason for age relation may be attributed to the increase in natural exposure to infection with time.

In children between 2-12 years of age, the immunity was found to be more significant as compared to the infants (0-2 years) in all the study groups. The highest IgG and IgM titres were 1:1280 and 1:640 in children (2-14 years) compared to only 1:40 for both IgG and IgM in infants (0-2 years).

A study carried out by McGregor et al in 1956 (91) led to the finding that malaria exerted its maximal effects in the first 18 months of life and that by the age of 3 years the unprotected children had developed a considerable immunity to the disease as witnessed by their ability to live asymptomatically with fairly dense parasitemia.

In a subsequent study on IFA response in Gambian population, McGregor et al (86) showed that the fluorescent antibody levels were high in newborns but declined in weeks following birth. Such titres remained low for the remainder first year of life and thereafter rose progressively throughout childhood into adult life. The studies of Collins

et al (92,93) in areas of Nigeria and Malaysia, which were hyperendemic for malaria, have also shown this increase in IFA response with age. The highest IFA titre of 1:160 in P. falciparum infections was shown among children upto 5 years of age and thereafter increasing to a 2-fold rise in titre value of 1 320 in children between 6-10 years of age In children 11 years and above a further 2-fold increase in titre value was observed in the results of Collins et al, yielding the highest titre of 1:640. The results of these findings are in agreement with the results of our study showing an increase in malaria antibody levels in children with age.

The IgM levels were highest in the slide-proven cases of vivax malaria This may be more likely because of the beginning of initial exposure to malaria in the lower age groups (2-5 years), although such a distinction has not been shown in the present study The initial exposure results in the production of IgM which precedes the appearance of IgG on primary encounter with a parasite (90).

Biswas and his colleagues carried out a survey on the natural occurrence of malarial antibodies in different regions of Northern India (53) In this age based survey, it was found that in District Mandla of Jabalpur (Madhya Pradesh) only 28% samples were detected positive for IgG, whereas 57% samples showed the presence of IgM, indicating

an active transmission of malaria in that area at the time of sample collection. They further found that in most individuals with active infections, IgM was detectable. But in persons with recent attack of the disease both IgM and IgG levels were elevated.

The observation documented by Tobie et al in 1966 (94) showed that the initial production of IgM followed by IgG antibody occurs rather early in response to the infection by Plasmodium. Again, these findings are in agreement with our results where IgM levels were highest at the time when parasitemia had risen to detectable levels.

The highest levels of IgG were recorded in children 15 to 90 days after the initial attack of vivax malaria. Brown et al in 1983 (95) reported that in majority of patients, convalescence was associated with an increase in antibody titre. Tobie et al (56) carried out a study on healthy American volunteers (with no previous malarial experience), by infecting them with bites of heavily infected Anopheles freeborni mosquitoes with P. vivax. The above workers showed that the maximum antibody titre was obtained from individuals 8 days after infection, and was maintained for approximately 3 weeks after which the titre had gradually begun to decline following chemotherapy.

Healthy children (2-14 years) were shown to possess higher IgG and IgM titres when compared to healthy infants (0-2 years). Similar report was provided in a study of Voller & Draper in 1982 (17). This study showed that in endemic areas virtually all children, aged 2 years and over, exposed to natural infection by Plasmodium had detectable levels of specific antibodies. Adults (15-56 years) were also included in this study. In these cases the levels of antibodies (IgG and IgM) were found to show a positive correlation with age. An illustration of the age related increase in the IgG levels among the healthy residents of the study area is given in Figure 3. Since malaria antibodies in a population reflect a cumulative experience of exposure to the parasite over the previous years, an age related increase in the titres is almost always expected. A study undertaken by Kumar et al in 1986 (96), to evaluate the role of seroepidemiology in the detection of malaria in and around Delhi also showed a definite trend towards age-related increase in serum titres of malarial antibodies.

Voller & Draper (17) further stated that a resident of a country where malaria is endemic and who is, or has been, repeatedly infected and who receives minimal treatment, is likely to develop higher levels of antibodies with a very wide spectrum of reactivity. This report was further confirmed by the extensive studies on Gambians

(85). The investigations showed that the prevalence and number of precipitin bands in the agar-gel diffusion test for malaria antibody detection increases with age, reflecting the exposure of the community and the development of immunity to malaria in adults of endemic areas. Similarly, in a study carried out in Nigeria by Molineaux and Gramiccia in 1980 (97), it was found that a positive association occurred between serology and parasitaemia, indicating exposure and infection while in older people a negative association occurred, indicating the development of immunity with age.

The findings of the present study relate well with above observations of the IgG levels in both slide-proven cases of vivax malaria and in healthy adult individuals, who were found to be similar. The overall IgG positivity however does not show a strongly positive correlation with the slide positivity for P. vivax in adults. But the antibodies of the IgM class showed a positive correlation with infected indiviudals with slide-proven vivax malaria.

Reinfection or relapse leads to a very rapid rise in antibody titres to even higher levels than those achieved in the initial infection and these levels persist for longer period (19,22,56,86). As expected, the individuals in this study with relapses and in whom post recovery follow-up tests were run also showed elevated levels of IgG reaching

upto a titre of 1:1280. However, some cases of relapses were found to have a higher titre of IgM in comparison to IgG levels (the actual data of relapse has not been shown of the individuals separately), indicating that the relapse may actually be a reinfection due to a heterologous strain of the parasite. More likely, it was not a relapse but reinfection with the same species but probably due to a different strain of the parasite.

IgM levels were found to be higher in most infected sera and a few negative sera from healthy individuals, denoting an active transmission of the disease in our study area during the time of sample collection.

IgA fraction of serum immunoglobulins was also detected in a few samples tested from all age groups of subjects with slide-proven vivax malaria, clinically suspected malaria (slide negative), as well as in post recovery cases and healthy individuals, randomly. A low titre level of specific IgA to P. vivax was detected, however its significance in malaria immunity is not clear.

The occurrence of IgA in response to infection by Plasmodium has been recorded in various studies. Tobie et al in 1966 (94) observed a little change in the specific IgA during P. vivax infections. Targett in 1970 (98) used class specific conjugates for IFA test in his study on falciparum

malaria in Gambia and found that sera of the residents had significant amounts of antibodies of IgG and IgM fractions with lesser amounts of IgA.

Collins et al in 1971 (99) also used immunoglobulin class-specific conjugates for IFA test and were able to measure antibody contents of IgG, IgM and IgA fractions separately. The IgM and IgA antibodies were found to be more transient, but IgG antibody was found to be much more persistent.

The significance of IgA in malaria infections is not yet clear. However in a study on animal models, Stechschulte et al in 1969 (100) found that most of the protective activity of hyperimmune rat serum resided in the IgA and IgG components of the 7 S immunoglobulin.

Finally, an observation was also made on a small scale, in which investigations pertaining to the effect of chemotherapy in slide-proven malaria were carried out (Table IV). These results indicate the decline in IgG levels after chemotherapy. IgM levels were found to be less affected in the same individual after chemotherapy. A report from Voller & Draper (17) is in agreement with our findings, indicating that antibodies decrease rapidly to undetectable levels in individuals following therapeutic cure.

In an earlier observation, Biswas *et al* (53) measured an increased level of IgM in acute infections and also in samples taken after antimalarial treatment in individuals living in villages of Ghaziabad (U.P.). However, the results of our study are somewhat contradictory to the findings of Biswas *et al*, who further indicated that elevated IgG levels were found in individuals with repeated attacks of malaria and also after the administration of chemotherapy.

4.2 CONCLUSION

The above study could thus be concluded as follows:

1. The study indicates that there is a positive correlation of the antibody levels increasing with age.

2. Also, the IgG levels do not show a positive correlation with the slide positivity of the individuals in the various age groups, whereas the IgM levels show a positive correlation with the slide positivity and also in people after treatment.

3. It was also found that high titres of IgM in the healthy adults were amply demonstrated, indicating the existence of significant numbers of *P. vivax* strains and hence proves the endemicity of the area.

4. From this study, it was further concluded that alongwith IgG, the detection of IgM antibodies by IFA tests can also be done in endemic populations.

The present study was an initial step in providing some idea of antibody profiles in cases of vivax malaria in the malaria endemic regions of the Aligarh District. It is more than obvious at this stage that finer details could be obtained through a similar study with a larger sample. The sample collection can thus be carried out over a longer time period to include the seasonal variation trends in malaria transmission. The resulting antibody levels can be compiled, areawise, from different parts of the state as well. This should provide some helpful baseline information which could be usefully employed for monitoring the various strategies brought into action for malaria control, and for other eradication programmes in the future. These findings would assume special significance when an asexual blood stage vaccine against malaria becomes available for use, whenever and wherever that may be possible.

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APPENDIX

1. PREPARATION OF THICK AND THIN BLOOD SMEARS FOR MICROSCOPICAL DIAGNOSIS OF MALARIA (101)

Specimen

Anticoagulated venous blood or non-anticoagulated capillary blood from skin puncture can be used. The parasites are usually most numerous in the blood towards the end of a malarial attack. The blood should always be collected before antimalarial drugs are administered.

(a) Thin Smear Preparation

A drop of blood is transferred on to a clean, grease free slide of 25 mm x 75 mm dimensions at about 15 to 20 cm away from the short edge of the slide. With a thumb and index finger of the left hand, the two edges of the slide are held lengthwise. The spreader slide is held with the right hand keeping an approximate angle of 30° to 45° between the two slides. The spreader is drawn back until it touches the drop of the blood. The blood is let to run along the edge of the spreader and the spreader is then pushed to the end of the slide with a smooth and quick movement. All the blood should be used before the spreader reaches the end of the slide. The spreader is kept between 30° to 45° throughout the process. A good slide should have the

smear occupy the central portion of the slide with clear margins on all sides of the slide. The smear is dried and fixed before staining.

(b) **Thick Smear Preparation**

A large drop of blood is applied on to a clean, grease free glass slide. Using the corner of a second slide or a coverslip, the drop of blood is spread over an area of about 1 cm, the smear should have even thickness and should appear translucent when wet. The smear is air dried for about 30 minutes before staining. Thick smears are not fixed.

2. **PROFORMA FOR BLOOD COLLECTION FOR MALARIA SEROLOGY**

Name :

Age/Sex :

Peripheral Blood Smear :

Positive - P. vivax/P. falciparum

Negative

Antimalarial Chemotherapy Administered : Yes/No

Relapse/Primary infection

Date/s of Previous Episode/s of Malaria (if any) :

Slide Results in Previous Malarial Attack :

Positive - P. vivax/P. falciparum

Negative

Any Chemotherapy Administered in the Previous Malarial Experience : Yes/No